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RESEARCH PAPER

Biocontrol efficacy of *Colletotrichum truncatum* for hemp sesbania (*Sesbania exaltata*) is enhanced with unrefined corn oil and surfactant

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In greenhouse and field experiments, an oil-in-water emulsion of unrefined corn oil and Silwet L-77 increased the biological weed control efficacy of *Colletotrichum truncatum* (Schw.) Andrus et Moore for control of the weed, hemp sesbania (*Sesbania exaltata* [Raf.] Rydb. ex A.W. Hill). The surfactant – corn oil emulsion stimulated germination and appressoria formation *in vivo* and *in vitro* and delayed the need for dew. We hypothesize that the corn oil protected the conidia from desiccation during the dew-free period and the surfactant stimulated spore germination and appressoria formation. In field experiments conducted over 3 years, a single application of a 50% (v/v) unrefined corn oil tank mixture containing 0.2% (v/v) Silwet L-77 surfactant controlled hemp sesbania in soybeans an average of 95%. Aqueous fungal suspensions or adjuvants alone did not visually affect or control hemp sesbania. The soybean yields were significantly higher in the plots where weeds were effectively controlled. These results suggest that formulating *C. truncatum* in unrefined corn oil and surfactant greatly increases its infectivity and the biocontrol potential of this pathogen.

Keywords: biocontrol agent, bioherbicide, *Colletotrichum truncatum*, oil-in-water emulsion, surfactant.

INTRODUCTION

Hemp sesbania (*Sesbania exaltata* [Raf.] Rydb. ex A.W. Hill) is a leguminous weed in soybean (*Glycine max* [L.] Merr.), cotton (*Gossypium hirsutum* L.), and rice (*Oryza sativa* L.), capable of reaching heights of 3 m at maturity (Lorenzi & Jeffery 1987). This weed ranks as one of the 10 most troublesome weeds in the three southern U.S. states of Arkansas, Louisiana, and Mississippi (Dowler 1992), reducing crop seed yield by shading and competition (King & Purcell 1997). Hemp sesbania is a prolific seed producer and can yield ≤ 21 000 seeds per plant (Norsworthy & Oliver 2000). Populations of 0.8–12.9 hemp sesbania plants m^{-2} emerging with soybean reduced the yield of soybean by ≤ 80% when allowed to interfere throughout the growing season (McWhorter & Anderson 1979). Emergence is characterized as quasi-simultaneous and, therefore, if a dense crop canopy is not formed soon after application of a postemergence herbicide that lacks residual control, weed re-infestation will probably result (Norsworthy & Oliver 2000).

The biological control of weeds by using plant pathogens has gained acceptance as a practical, safe, and environmentally beneficial weed management method applicable to agro-ecosystems (Charudattan 2005). In order to be even more predictable and acceptable, it is necessary to express the maximum capability of a bioherbicidal pathogen to infect, kill or reduce the competitiveness of the weed host (Hallett 2005). Research has indicated that the fungus, *Colletotrichum truncatum* (Schw.) Andrus et Moore, has promise as a bioherbicide for controlling hemp sesbania (Boyette 1991; Boyette *et al.* 1993; Abbas & Boyette 2000). However, as is the case with most foliar pathogens, the spores (conidia) of this fungus require a dew period to germinate, establish infection, and cause disease (Boyette 1991; Boyette *et al.* 1993). Free-moisture events are difficult to predict in the field, thus

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knowledge of the effects of several suboptimal dew events, as opposed to a single event, is important in predicting bioherbicidal efficacy (Walker & Boyette 1986; Weidemann *et al.* 1995). Increasing the infectivity (the ability to infect a host) of a bioherbicidal fungus in any given dew period theoretically will increase the effectiveness of the pathogen (Yang & TeBeest 1993). Previous research in our laboratory and elsewhere has shown that invert (water-in-oil) emulsions and various vegetable oil-in-water emulsions can retard evaporation and trap water in bioherbicide spray mixtures, thereby decreasing the amount of additional free moisture required to initiate spore germination and infection (Quimby *et al.* 1989; Winder & van Dyke 1990; Amsellen *et al.* 1991; Mintz *et al.* 1992; Auld 1993; Boyette *et al.* 1993; Boyette 1994; Egley & Boyette 1995; Sandrin *et al.* 2003). For example, greenhouse and field results indicated that excellent control (>95%) of sicklepod (*Senna obtusifolia* L.) with the fungus, *Alternaria cassiae* Jurair et Khan, could be achieved with little or no dew when lecithin was used as an emulsifying agent, and paraffin oil and wax were used to retard water evaporation (Quimby *et al.* 1989). Similarly, it was shown that hemp sesbania could be effectively controlled in soybean by *C. truncatum* spores formulated in a water-in-oil invert emulsion applied using specialized spraying equipment, such as air-assist nozzles (Boyette *et al.* 1993). Although the invert formulation provided excellent hemp sesbania control (>90%), the difficulty in applying this viscous mixture precluded its practical usage. Therefore, another effective, simple formulation that could be applied with conventional spraying equipment was sought.

We have previously reported in greenhouse experiments that oil-in-water emulsions of unrefined corn oil and *C. truncatum* spore suspensions reduced the dew period requirements for maximum weed infection and the mortality of hemp sesbania from 12 h to 2 h, and delayed the need for free moisture for greater than 72 h (Boyette 1994). We have also demonstrated that unrefined corn oil, but not refined corn oil, stimulated the germination of *C. truncatum* spores (Egley & Boyette 1995). Other refined oils were likewise unstimulatory (Egley & Boyette 1995). Research has also shown that the addition of the surfactant, Silwet L-77, to an unrefined corn oil emulsion promoted the germination and infectivity of *Alternaria helianthi* spores on common cocklebur (*Xanthium strumarium* L.) (Abbas & Egley 1996). The purpose of the present research was to examine the effects of an unrefined corn oil – water emulsion containing Silwet L-77 surfactant on *C. truncatum* spore germination, appressoria formation, delayed dew under controlled environmental conditions, weed control efficacy, and effects on soybean yields of *C. truncatum* in this formulation under field conditions.

MATERIALS AND METHODS

Laboratory and greenhouse experiments

Maintenance of the fungus

A single strain of *C. truncatum* (NRRL 18434; Agricultural Research Service Patent Culture Collection, Peoria, IL, USA) was used in all experiments. The fungus was preserved in screw-capped tubes containing sterilized soil (Bakerspigel 1953). The cultures were grown for 5–7 days on potato dextrose agar (PDA; Difco Laboratories, Detroit, MI, USA) in 10 cm plastic Petri dishes that were incubated on the open-mesh wire shelves of an incubator (I-35LLVL; Percival Scientific, Perry, IA, USA) at 25°C under cool, white fluorescent lighting (12 h photoperiod). The spores were harvested by rinsing the cultures with water and straining through double-layered cheesecloth. The spore densities were determined with hemocytometers (Thermo Fisher Scientific, Waltham, MA, USA) and dilutions were made to give the desired inoculum concentrations. In experiments that included unrefined corn oil, a 1:1 ratio with water was utilized. The emulsion formulations were prepared by adding unrefined corn oil (Spectrum Naturals, Petaluma, CA, USA) to water. The mixtures were thoroughly mixed with a vortex mixer (Vortex Genie, New York, USA).

Effects of adjuvants on conidia germination and appressorium formation in vitro

Appressoria formation by *C. truncatum*, as well as by other *Colletotrichum* spp., is crucial to the establishment of infection (Emmett & Parbery 1975). The *in vitro* effects of these adjuvants upon spore germination and appressoria formation were measured by placing 0.1 μL droplets of unrefined corn oil – surfactant emulsion spore suspensions that contained $\approx 1 \times 10^6$ spores ml^{-1} on glass microscope slides (three droplets per plate) and incubating the slides on water-saturated filter paper (Whatman International, Middlesex, UK) in plastic Petri plates (relative humidity, RH = 100%) at 25°C in alternating 12 h light/12 h dark regimens. The light intensity at dish level was ≈ 200 μmol L^{-2} s^{-1}. The microscopic counts of the germinated conidia were made after 8 h. The treatments utilized in these studies were as follows: (i) *C. truncatum* spores in water suspension; (ii) *C. truncatum* spores in unrefined corn oil (1:1); (iii) *C. truncatum* spores in 0.2% (v/v) Silwet L–77 (polyalkyeneoxide-modified heptamethyltrisiloxane;

Loveland Industries, Greeley, CO, USA) – water; and (iv) *C. truncatum* spores in unrefined corn oil and 0.2% (v/v) Silwet L–77.

Effects of adjuvants and dew treatment on conidia germination and appressorium formation and infectivity in vivo

Hemp sesbania plants were grown from seeds collected by the authors from plants outside of cultivation in Stoneville, Mississippi, USA, in a commercial potting mix (Jiffy Mix 60510; Jiffy Products, Batavia, IL, USA) contained in peat strips. Each strip contained 12 plants. The potting mix was supplemented with a controlled-release fertilizer (14:14:14, nitrogen : phosphorus : potassium; Osmocote; Grace Sierra Horticultural Products, Milpitas, CA, USA).

The plants were placed in subirrigated trays that were mounted on greenhouse benches. The greenhouse temperatures ranged from 25–30°C with 40–90% RH. The photoperiod was 12 h with 1650 μmol m^{-2} s^{-1}, measured at 12.00 hours. The hemp sesbania seedlings were sprayed until run-off (≈ 150 L ha^{-1}) at a concentration of 1×10^7 conidia mL^{-1} and were placed in the greenhouse (28–33°C, 60–80% RH, ≈ 12 h day length) for 24 h before transfer to a dew chamber (100% RH, 25°C) for 8 h (delayed dew). Other groups of plants received either an immediate dew treatment (immediate dew) as described or no dew treatment (no dew). Eight leaflets (approximate leaflet size: 20 mm × 8 mm) were randomly collected from different seedlings in each replication of each treatment and placed on a strip of wet filter paper on a glass microscope slide. The conidia were stained by spreading a few drops of lactophenol cotton blue over the leaf surface. The first 200 spores on the leaves in each replication were observed under ×400 magnification and were scored for spore germination and appressoria formation. The appressoria results were expressed as a percentage of the germinated conidia. After the dew treatment, the seedlings were returned to the greenhouse and observed for disease development for 14 days. Weed control was visually assessed on a 10-point scale, where healthy plants were scored "0" and completely necrotic plants were scored "10".

Statistical analysis

In all experiments, the treatments were arranged in a randomized block design with four replicates. The greenhouse and laboratory experiments were repeated over time. The observations from the repeated experiments were pooled. The means were subjected to analysis of variance and then compared with Fisher's Least Significant Difference (LSD) test ($P = 0.05$) when the F-test from the analysis indicated significance.

Field experiments

The field experiments were conducted on a Dundee, very fine sandy loam (Aeric Ochraqualf, 24% clay, 29% sand, 47% silt, 1.2% carbon, pH = 6.3) from 1996 to 1998 at the Southern Weed Science Experimental Farm, Stoneville, MS, USA. The test plots consisted of four rows of "Centennial" cv. soybeans (Hartwig & Epps 1977), 12.2 m long and 1 m apart, with the two center rows receiving treatment. All rows were planted with scarified hemp sesbania seed at a density of ≈ 100 seeds m^{-1} of row. The treatments consisted of: (i) untreated; (ii) fungus only; (iii) 0.2% Silwet L–77 surfactant only; (iv) 50% (v/v) unrefined corn oil – water only; (v) fungus in 0.2% Silwet L–77 surfactant; (vi) fungus in 50% (v/v) unrefined corn oil; (vii) fungus in 0.2% Silwet L–77 surfactant and 50% (v/v) unrefined corn oil; (viii) acifluorfen (1.8 kg ai ha^{-1}); and (ix) a hand-weeded check. The spray applications were made with hand-held pump sprayers at a rate of ≈ 150 L ha^{-1}. The inoculum concentrations were 1.0×10^7 conidia mL^{-1} in those treatments receiving a fungal component. The planting dates were 14 May 1996, 20 May 1997, and 12 June 1998. The treatments were made when weed seedlings were in the first-to-third true leaf stages (≈ 2 weeks after planting). The percentage control of the hemp sesbania was determined in randomly selected 3.0 m × 0.46 m areas at 8 day intervals for 28 days. The weed mortality was based on a 0–100% rating based on the numbers of plants that were killed or severely damaged within each quadrant. The experiments were arranged as randomized complete block designs with four replications. The data were tested for homogeneity (Steel *et al.* 1997) and pooled over the 3 year testing period and analyzed using the analysis of variance. The percentages of hemp sesbania controlled received arc sin transformation prior to analysis. The treatment means were separated by Fisher's LSD test at the 0.05 level of probability.

RESULTS AND DISCUSSION

Effects of adjuvants, conidia germination, and appressorium formation *in vitro*

Silwet L-77 surfactant and unrefined corn oil, alone and in combination with the surfactant, stimulated spore germination and appressoria formation on the glass slides (Table 1). The spores germinated at a rate of 78% in the emulsified unrefined corn oil while < 1% germinated in water alone; these were typical results for these treat-

Table 1. Effects of unrefined corn oil – Silwet L-77 surfactant emulsion upon germination and appressoria formation of *Colletotrichum truncatum* spores *in vitro*

Treatment	Germination (%)†	Appressoria formation (%)
Distilled water	<1	0.0
Unrefined corn oil‡	78	20.0
Silwet L-77§	84	18.0
Unrefined corn oil + Silwet L-77	88	24.0
LSD_{05}	6	6.1

† Germinated conidia were determined by microscopic count after 24 h incubation on hemp sesbania leaflets. The conidia were suspended in distilled water; unrefined corn oil was added to make a 1:1 (v/v) emulsion; ‡ unrefined corn oil was added to make a 1:1 unrefined corn oil/aqueous conidia emulsion; § the conidia were suspended in distilled water; Silwet L–77 was added to yield a 0.2% solution. LSD, least significant difference.

ments (Egley & Boyette 1995). Spore germination was significantly improved (84% and 88%, respectively) for the surfactant only and unrefined corn oil – surfactant treatments (Table 1). Appressoria formation was also significantly improved by Silwet L-77 and unrefined corn oil (Table 1).

Effects of adjuvants, conidia germination, and appressorium formation *in vivo*

Unrefined corn oil emulsions enhanced conidia germination and appressoria formation on hemp sesbania in the greenhouse, even when the plants received no dew or after a dew delay (Figs 1,2). Silwet L–77 alone increased spore germination and appressoria formation when a dew treatment occurred, but not in the absence of dew or with a delayed dew treatment (Figs 1,2). A combination of unrefined corn oil and surfactant resulted in slightly improved spore germination and appressoria formation after an immediate dew treatment and significantly improved germination and appressoria formation in the absence of dew or with a delayed dew treatment (Figs 1,2). These improved rates of germination and appressoria formation resulted in higher levels of disease in the hemp sesbania, especially in treatments including unrefined corn oil and delayed or no dew periods. Unrefined corn oil with a delayed dew period was able to provide comparable control levels to an immediate dew period (Fig. 3).

Field experiments

Of the fungal treatments tested, *C. truncatum* spores formulated in the unrefined corn oil with or without

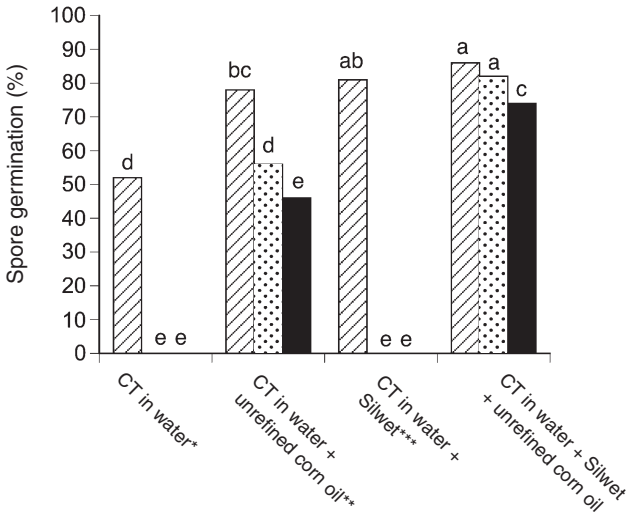

Fig. 1. *In vivo Colletotrichum truncatum* spore germination. **Colletotrichum truncatum* (CT); **unrefined corn oil was added to make a 1:1 unrefined corn oil/aqueous conidia emulsion; ***the conidia were suspended in distilled water; Silwet L–77 was added to yield a 0.2% solution. Bars with the same lowercase letter are not significantly different by Fisher's mean separation test ($P = 0.05$). (▨), immediate dew; (⁙), delayed dew; (■), no dew.

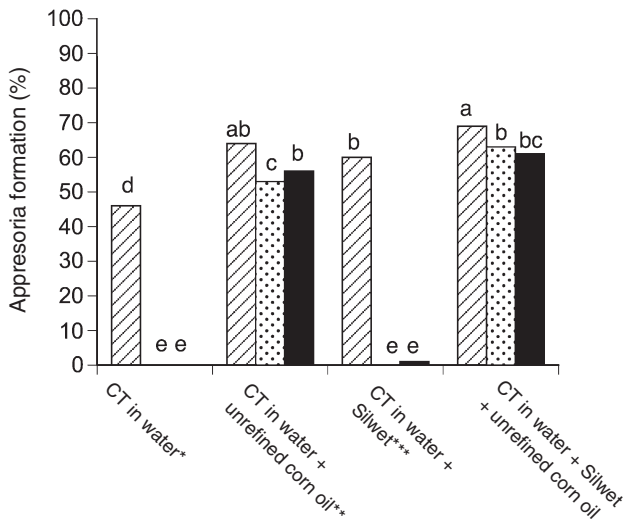

Fig. 2. *In vivo Colletotrichum truncatum* appresoria formation. **Colletotrichum truncatum* (CT); **unrefined corn oil was added to make a 1:1 unrefined corn oil/aqueous conidia emulsion; ***the conidia were suspended in distilled water; Silwet L–77 was added to yield a 0.2% solution. Bars with the same lowercase letter are not significantly different by Fisher's mean separation test ($P = 0.05$). (▨), immediate dew; (⁙), delayed dew; (■), no dew.

Table 2. Biological control of hemp sesbania under field conditions, Stoneville, MS, USA†

Treatment	Weed mortality (%)	Biomass reduction (%)	Soybean yield (kg ha^{-1})
Untreated	0d	0d	1288c
Colletotrichum truncatum only‡	0d	0d	1296c
C. truncatum/Silwet L-77§	22c	40c	1909b
C. truncatum/unrefined corn oil¶	88b	92ab	2666a
C. truncatum/unrefined corn oil/Silwet L-77††	95ab	96a	2790a
Acifluorfen	100a	100a	2856a
Weed-free check	100a	100a	2888a

† Means followed by the same letter do not differ at $P = 0.05$ according to Duncan's Multiple Range Test; ‡ inoculum rate: 1×10^7 conidia mL^{-1}; § Silwet L-77 was added to make a 0.2% solution; ¶ unrefined corn oil was added to make a 1:1 (v/v) emulsion; †† Silwet L-77 was added to a 1:1 unrefined corn oil/aqueous conidia emulsion to yield a 0.2% solution.

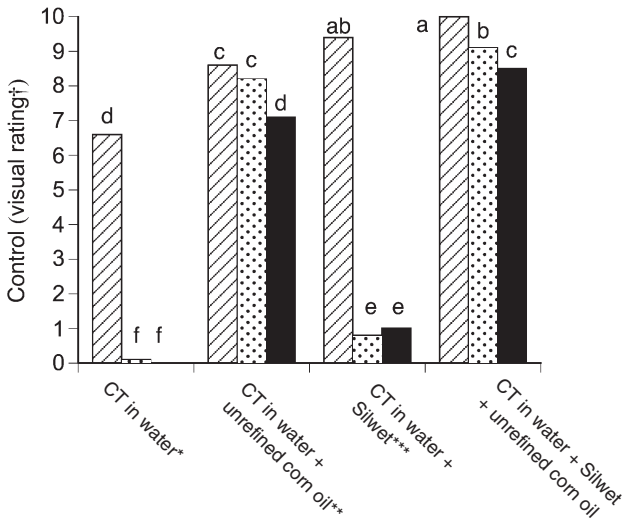

Fig. 3. Control of hemp sesbania by *Colletotrichum truncatum*. †Control was determined from a visual rating scale in which healthy plants were scored "0" and completely necrotic plants were scored "10"; **C. truncatum* (CT); **unrefined corn oil was added to make a 1:1 unrefined corn oil/aqueous conidia emulsion; ***the conidia were suspended in distilled water; Silwet L–77 was added to yield a 0.2% solution. Bars with the same lowercase letter are not significantly different by Fisher's mean separation test ($P = 0.05$). (▨), immediate dew; (⊡), delayed dew; (■), no dew.

surfactant treatments effectively controlled hemp sesbania (Table 2). Hemp sesbania was controlled by 95% after 8 days with a single fungus – corn oil – surfactant treatment (Table 2). This level of control was not significantly different from the control achieved with acifluorfen or the weed-free check (Table 2). Likewise, soybean yields were not significantly different from either the acifluorfen or weed-free treatments (Table 2).

It was concluded that the unrefined corn oil helped to maintain the virulence of the conidia on the plant surface during a dew-free period. The corn oil may have protected the conidia from desiccation by substituting for, or supplementing, the matrix normally produced by the conidia. It was also concluded that the unrefined corn oil stimulated conidial germination when a dew period occurred. Thus, the unrefined corn oil has potential as a formulation to enable this mycoherbicide to persist on the target weed without a dew period and to germinate and infect the weed when moisture in the form of dew becomes available. The unpredictability of dew periods and the resulting inconsistent level of weed control are major factors limiting the use of mycoherbicides in agriculture (Greaves & MacQueen 1988). In the present studies, the addition of Silwet L-77 to unrefined corn oil – *C. truncatum* spore suspensions resulted in increased mortality to hemp sesbania. Silwet L-77 has been reported to provide enhanced wetting of the plant foliage and to increase stomatal infiltration of aqueous solutions in several different plant species (Field & Bishop 1988; Zabkiewicz & Gaskin 1989). The extremely low oil – water surface tension (20 dynes cm^{-1}) created by Silwet L-77 has been shown to facilitate the direct penetration by bacterial cells of *Pseudomonas syringae* pv. *phaseolicola* van Hall into kudzu (*Pueraria lobata* L.) stomata, thereby enhancing infection by this bacterial pathogen (Zidak *et al.* 1992). However, this is not likely to occur with *C. truncatum* and hemp sesbania, since previous research has shown that hemp sesbania stomatal openings are not large enough to accommodate direct penetration by fungal spores that are in the size range of *C. truncatum* spores (Van Dyke & Trigiano 1987). A more likely explanation for the increased infectivity and mortality to hemp sesbania is the stimulation of spore germination and appressoria by the adjuvants used in these studies (Table 1; Figs 1,2).

Previous research has shown that there is a direct correlation between unrefined corn oil concentrations and appressoria formation, with a 1:1 unrefined corn oil: aqueous spore suspension of *C. truncatum* the optimal ratio (Egley & Boyette 1995). Soybean and sunflower oils increased the level of infection of northern jointvetch (*Aeschynomene virginica*) plants by *Colletotrichum gloeosporioides* f. sp. *aeschynomene* (Sandrin *et al.* 2003). The inoculation of seedlings with fungal spore suspensions in these oils resulted in more disease than when they were inoculated with suspensions of spores in water alone. The lengths of the dew periods required to establish equivalent levels of disease by spore suspensions containing soybean or sunflower oil were ≈ 4–8 h less compared to aqueous suspensions. Spore germination and appressoria formation were unaffected by either of the oils tested in the *in vitro* assays; however, 10% soybean oil and 10% sunflower oil increased spore germination in comparison to spores that were suspended in water. It is not known if appressoria formation could be enhanced by increasing the vegetable oil: aqueous *C. truncatum* component. The mechanism(s) of these pathogen: oil: surfactant interactions are complex and will require research beyond the scope of this study. Although the primary role of cuticular waxes is to protect the plant from desiccation (Reed & Tukey 1982), waxes are one of the physicochemical barriers involved in plant defense against bacterial and fungal pathogens (Jenks *et al.* 1994). Cuticular waxes create a hydrophobic surface, and various oils, surfactants, and adjuvants can act as solvents to dissolve or disrupt these waxes. Thus, if corn oil and Silwet break down hydrophobic barriers, pathogen entry could be facilitated, especially considering that *C. truncatum* spores are hydrophilic (Boyette *et al.* 1993).

Results from the field trials of two pathogens, *Bipolaris sacchari* and *Dreshlera gigantea*, indicated that high volumes of spore: oil emulsions were necessary to achieve effective levels of foliar blighting (Yandoc *et al.* 2005). For a weed such as hemp sesbania, which can produce numerous leaves during a growing season and grow to a height of 2 m, a high application rate is needed to ensure complete coverage of the massive amount of foliage. Complete coverage of the leaf and stem surfaces with inoculum also is important because secondary inoculum production by *C. truncatum* is limited (Boyette *et al.* 1993) and possibly infects emerging hemp sesbania seedlings as a soil-borne pathogen by production of microsclerotia (Schisler & Jackson 1996). The uniform distribution of inoculum on the plant surface sufficiently early in the season is essential for creating epidemics (Shrum 1982). This requirement for large volumes of inoculum to assure biocontrol efficacy also has been demonstrated by other studies (Mortensen & Makowski 1989; Klein & Auld 1995; Imaizumi *et al.* 1997). Imaizumi *et al.* (1997) determined that there is a distinct dose – response effect of *Xanthomonas campestris* (Pammel) Dowson pv. *poae* (strain JT-P482) cell concentration and carrier (water) volume on the level of annual bluegrass (*Poa annua* L.) control. Klein and Auld (1995) reported that high water volumes favored the development of disease caused by *Colletotrichum orbiculare* (Berk. et Mont.) Arx on spiny cocklebur (*Xanthium spinosum* L.). However, they also observed that when the environmental conditions were conducive to disease development, a lower number of spores and lower carrier volumes were adequate. Mortensen and Makowski (1989) observed that the application of *Colletotrichum gloeosporioides* (Penz.) Penz. et Saccf. sp. *malvae* in a higher volume of water produced a more uniform initial infection on round-leaved mallow (*Malva pusilla* Sm.) in the field. The results in this report indicate that, when properly formulated and applied at critical times, *C. truncatum* can be a very effective bioherbicide for controlling hemp sesbania.

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